**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

ATTY.'S DOCKET: IKEGAMI=2

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| In re Application of: | ) | Group Art Unit: 1638 |
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| Hakuo IKEGAMI et al. | ) | Examiner: G. L. HELMER |
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| Appln. No.: 09/893,005 | ) | Washington, D.C. |
| | ) | |
| Date Filed: June 28, 2001 | ) | Confirmation No. 6398 |
| | ) | |
| For: TRANSGENIC PLANTS | ) | July 14, 2005 |

**DECLARATION UNDER 37 CFR 1.132**

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

1. I, Shigeharu FUKUDA, declare as follows:

2. I am a citizen of Japan, residing at 2189, Atsu, Okayama-shi, Okayama, Japan.

3. In 1976, I received a bachelor of Agriculture from Osaka Prefecture University, and in 1984 I received a doctorate of Agriculture from the above-identified university.

4. As shown in my curriculum vitae attached hereto as Attachment A, from 1976 to 1980, I researched in Hayashibara Co., Ltd., fundamental studies and industrial applications of carbohydrates and their related enzymes. From 1981 to 1995, I researched and directed in Hayashibara Biochemical Laboratories,

Inc., fundamental studies and industrial applications of physiologically active substances, particularly, interferons and interleukins. Since 2001, I have been a corporate director of Hayashibara Biochemical Laboratories, Inc.

5. I have read and am thoroughly understood the present invention and the content of the United States Patent No. 4,956,282, titled "Mammalian peptide expression in plant cells", applied for by Calgene, Inc., Davis, Calif., cited in an official action in the procedure of the present invention.

6. To demonstrate a significant difference of the expression level of interferon-α between the transgenic plants according to the present invention and transgenic tobacco plants including the one in the above-identified patent, I conducted the following Experiments.

7. Transgenic plants according to the present invention

Experiment 7-1: Transgenic spinach plant

According to the method disclosed in Example 1 of the specification of the present invention, spinach plant (*Spinacia oleracea*) leaves were transformed with *Agrobacterium*, into which had been introduced a human interferon-α2 gene registered at the nucleic acid database "GenBank®" under the accession No. Y11834, and then incubated in conventional MS agar medium containing cefotaxime, kanamycin and auxin in an incubator for plants under conditions of a daily cycle of successive 16-hour light (1,000 lux illumination) and successive 8-hour darkness

at 25°C for three weeks.

The resulting rooted spinach leaves were transferred unto conventional auxin-free MS agar medium and incubated in the incubator similarly as above to regenerate juvenile plant bodies. Thereafter, respective juvenile plant bodies were partly cut off, and the resultant specimens were homogenized and checked whether they contained the human interferon-α2 gene by using conventional PCR method.

The juvenile plant bodies, which had been proved to have the human interferon-α2 gene, were selected and cultured until fully grown up in a greenhouse controlled at a temperature of 17°C and a relative humidity of 50 to 60% under conditions of a daily cycle of successive 16-hour light (5,000 lux illumination) and successive 8-hour darkness. The leaves were harvested from 100 transgenic spinach plants and cut into pieces, and one kilogram of the resulting pieces was sampled, homogenized, and extracted with 10 L of phosphate buffered saline (pH 8.0). The extract was centrifuged (10,000 x 60 min) to obtain a supernatant which was then concentrated by ultrafiltration. The concentrate was purified by usual methods and subjected to the following assay.

The anti-viral activity of the purified preparation was assayed by using a system of human FL cells and Sindbis virus, revealing that the above supernatant had about 17 international units of anti-viral activity per milliliter.

Based on the activity, it was revealed that the transgenic spinach plant expressed human interferon-α2 in an amount of about 1.5 μg/kg fresh weight.

Experiment 7-2: Transgenic celery plant

Similarly as the method in the above Experiment 7-1, juvenile transgenic celery plants (*Apium graveolens*) were constructed and cultured until fully grown up in a greenhouse controlled at a temperature of 20°C and a relative humidity of 50 to 60% under conditions of a daily cycle of successive 16-hour light (5,000 lux illumination) and successive 8-hour darkness. The 10 transgenic celery plants were removed their roots and cut into pieces, and one kilogram of the resulting pieces was homogenized, and extracted with 10 L of phosphate buffered saline (pH 8.0). Similarly as in Experiment 7-1, the extract was centrifuged to obtain a supernatant, which was then purified and assayed for anti-viral activity, revealing that the above supernatant had about 25 international units of anti-viral activity per milliliter.

Based on the activity, it was revealed that the transgenic spinach plant expressed human interferon-α2 in an amount of about 2.2 μg/kg fresh weight.

Experiment 7-3: Transgenic cabbage plant

Similarly as the method in the above Experiment 7-1 except for using a human interferon-α8 gene registered at the nucleic acid database "GenBank®" under the accession No. X03125 in place of the human interferon-α2 gene, juvenile transgenic cabbage plants (*Brassica oleracea var. capitata*) were constructed and cultured until fully grown up in a greenhouse controlled at a temperature of 15°C and a relative humidity of 50 to 60% under conditions of a daily cycle of successive 16-

hour light (5,000 lux illumination) and successive 8-hour darkness. The 10 transgenic cabbage plants were removed their roots and cut into pieces, and one kilogram of the resulting pieces was sampled and homogenized by a homogenizer and extracted with 10 L of phosphate buffered saline (pH 8.0). Similarly as in Experiment 7-1, the extract was centrifuged to obtain a supernatant and assayed for anti-viral activity, revealing that the supernatant had about 20 international units of anti-viral activity per milliliter.

Based on the activity, it was revealed that the transgenic cabbage plant expressed human interferon-α8 in an amount of 1.8 μg/kg fresh weight.

Experiment 7-4: Transgenic potato plant

Similarly as the method in the above Experiment 7-1 except for using a human interferon-α8 gene registered at the nucleic acid database "GenBank®" under the accession No. X03125 in place of the human interferon-α2 gene and using a potato plants (*Solanum tuberosum*), juvenile transgenic potato plants were constructed and cultured until fully grown up in a greenhouse controlled at a temperature of 18°C and a relative humidity of 50 to 60% under conditions of a daily cycle of successive 16-hour light (5,000 lux illumination) and successive 8-hour darkness. One kilogram of potatos and leaves was collected from 10 transgenic potato plants, homogenized, and extracted with 10 L of phosphate buffered saline (pH 8.0). Similarly as in Experiment 7-1, the resulting extract was centrifuged to obtain a supernatant, which was then purified and

assayed for anti-viral activity, revealing that the supernatant had about 25 international units of anti-viral activity per milliliter.

Based on the activity, it was revealed that the transgenic potato plant expressed human interferon-α8 in an amount of 2.0 μg/kg fresh weight.

8. Transgenic tobacco plant as a control

Experiment 8-1: Control 1

In accordance with the method in the above Experiment 7-1, juvenile transgenic tobacco plants (*Nicotiana tabacum xanthii*), into which had been introduced a human interferon-α2 gene registered at the nucleic acid database "GenBank®" under the accession No. Y11834, were constructed and cultured until fully grown up in a greenhouse controlled at a temperature of 20°C and a relative humidity of 50 to 60% under conditions of a daily cycle of successive 16-hour light (5,000 lux illumination) and successive 8-hour darkness.

The leaves were collected from 10 transgenic tobacco plants and cut into pieces, and one kilogram of the resulting pieces was homogenized and extracted with 10 L of phosphate buffered saline (pH 8.0). Similarly as in the above Experiment 7-1, the extract was centrifuged, purified, and assayed for anti-viral activity, revealing that the supernatant had an activity of about 0.1 international unit of anti-viral activity per milliliter.

Based on the activity, it was revealed that the transgenic tobacco plant expressed human interferon-α2 in an

amount of about 0.01 μg/kg fresh weight.

Experiment 8-2: Control 2:

Tobacco callus was obtained in accordance with the method in the United States Patent No. 4,956,282 except for using either a human interferon-α2 gene registered at the nucleic acid database "GenBank®" under the accession No. Y11834, or a human interferon-α8 gene registered at the nucleic acid database "GenBank®" under the accession No. X03125, in place of the murine γ-interferon gene used in the above-identified patent.

The callus was proliferated according to the method disclosed in Example 1 of the specification of the present invention to obtain juvenile transgenic tobacco plants (*Nicotiana tabacum xanthii*), into which had been introduced the human interferon-α2 gene or the human interferon-α8 gene.

The juvenile transgenic tobacco plants were cultured similarly as in Experiment 7-1 to obtain transgenic tobacco plants capable of producing human interferon-α2 or interferon-α8.

For each type of the transgenic tobacco plants, the leaves were collected from 10 transgenic tobacco plants and cut into pieces, and one kilogram of the resulting pieces was homogenized and extracted with 10 L of phosphate buffered saline (pH 8.0). Similarly as in the above Experiment 7-1, the extract was centrifuged, purified, and assayed for anti-viral activity.

As a result, the two-types of transgenic tobacco plants thus obtained expressed substantially the same level of

human interferon-α2 or interferon-α8 as that of the transgenic tobacco plants in Experiment 8-1.

9. Conclusion:

As evident from the above Experimental results, human interferon-α, i.e., interferon-α2 and interferon-α8 were expressed in the transgenic spinach plant, celery plant, cabbage plant, and potato plant according to the present invention in a yield of about 150-folds higher than those in transgenic tobacco plants. This indicates that the transgenic plants according to the present invention are superior to transgenic tobacco plants in expression level when expressing human interferon-α in grown up plant bodies.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Shigeharu Fukuda

NAME: Shigeharu FUKUDA

14th day of July, 2005

DATE: 14th day of July, 2005

# CURRICULUM VITAE

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| Name: | Shigeharu FUKUDA, formerly Shigeharu YOSHIKAWA before marriage |
| Affiliation: | Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc., 675-1, Fujisaki, Okayama, Japan 702<br>Tel:+81 86 276 3141 |
| Date of Birth: | February 29, 1952 |
| Education: | Graduated and received a bachelor from Osaka Prefecture University, Agricultural Department in 1976. Received a doctorate of Agriculture at Osaka Prefecture University in 1984. |

Brief Chronology of Employment:

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| 1976 (April) | Researcher, Hayashibara Co., Ltd. |
| 1976 (from May) | Researcher, Osaka Industrial Research Association, under the employment of Hayashibara Co., Ltd. |
| 1980 | Researcher, Hayashibara Co., Ltd. |
| 1981 | Researcher, Hayashibara Biochemical Laboratories, Inc. |
| 1995 | Director of Laboratory of Amase Institute, Hayashibara Biochemical Laboratories, Inc. |
| 2001 | Corporate Director, Hayashibara Biochemical Laboratories, Inc. |
| 2004 | Director of Laboratory of Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc. |

Public Employment:

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| 1989-1998 | Manager of FCCA (Forum: Carbohydrates Coming of Age)<br><br>Member of Editorial Board of TIGG (Trends in Glycoscience and Glycotechnology) |
| 1995-1997 | Affiliate Professor, Cooperative Research Center, Okayama University |

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| 2005- | Steering Committee, Chugoku Technology Promotion Organization; |
| | Commissioner of Osaka Industrial Research Association, Osaka Municipal Technical Research Institute; |
| | Board of Japan Society for Bioscience, Biotechnology, and Agrochemistry; |
| | Part-time Assistant Professor of Faculty of Engineering, Okayama University |

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